Analysis of Genomic DNA by Southern Hybridization

Localization of particular sequences within genomic DNA is usually accomplished by the transfer techniques described by Southern (1975). Genomic DNA is digested with one or more restriction enzymes, and the resulting fragments are separated according to size by electrophoresis through an agarose gel. The DNA is then denatured in situ and transferred from the gel to a solid support (usually a nitrocellulose filter or nylon membrane). The relative positions of the DNA fragments are preserved during their transfer to the filter. The DNA attached to the filter is hybridized to radiolabeled DNA or RNA, and autoradiography is used to locate the positions of bands complementary to the probe. The techniques described below are suitable for analysis of restriction digests of mammalian genomic DNA. However, they can easily be adapted to accommodate restriction digests of plasmids, cosmids, and λ bacteriophages.

The amount of genomic DNA needed to generate a detectable hybridization signal depends on a number of factors, including the proportion of the genome that is complementary to the probe, the size of the probe and its specific activity, and the amount of genomic DNA transferred to the filter. Under the best conditions, the method is sufficiently sensitive to detect, in a autoradiographic exposure of several days, less than 0.1 pg of DNA complementary to a probe that has been radiolabeled with ^{32}P to high specific activity ($> 10^9$ cpm/μg; see Chapter 10). A sequence of 1000 bp that occurs only once in the mammalian genome (i.e., 1 part in 3 million) can be detected in an overnight exposure if 10 μg of genomic DNA is transferred to the filter and hybridized to a probe several hundred nucleotides in length. Because the strength of the signal is proportional to the specific activity of the probe and inversely proportional to its length, Southern hybridization reaches the limits of its sensitivity when very short probes are used. To obtain a detectable signal with oligonucleotide probes, it is therefore necessary to radiolabel oligonucleotides to the highest specific activity possible, to increase the amount of target DNA on the filter, and to expose the autoradiograph for several days.

SEPARATION OF RESTRICTION FRAGMENTS OF MAMMALIAN GENOMIC DNA BY AGAROSE GEL ELECTROPHORESIS

1. Digest an appropriate amount of DNA with one or more restriction enzymes. For Southern analysis of mammalian genomic DNA, approximately 10 μg of DNA should be loaded into each slot of the gel when probes of standard length (>500 bp) and high specific activity ($>10^9$ cpm/μg) are used to detect single-copy sequences; 30–50 μg of DNA are needed when oligonucleotides are used as probes. Proportionately less DNA may be used when the sample contains higher concentrations of the sequences of interest.

   When handling high-molecular-weight genomic DNA, use either pipette tips that have been cut off with a sterile razor blade to enlarge the orifices or disposable wide-bore capillaries.

   The concentrations of DNA in preparations of high-molecular-weight mammalian genomic DNA are often so low that it is necessary to carry out restriction digests in relatively large volumes. After digestion is complete, the fragments of DNA are concentrated by precipitation with ethanol and are applied to the gel in a small volume of gel-loading buffer (see step 2). However, it is essential to ensure that the ethanol is removed from the DNA solution before it is loaded on the gel. If significant quantities of ethanol remain, the DNA "crawls" out of the slot of the gel. Heating the solution of redissolved DNA to 70°C in an open tube for 10 minutes is usually sufficient to drive off most of the ethanol. This treatment also disrupts base pairing between cohesive termini of restriction fragments.

   The chief problem encountered during digestion of high-molecular-weight DNA is unevenness of digestion caused by variations in the local concentrations of DNA. Clumps of DNA are relatively inaccessible to restriction enzymes and can be digested only from the outside. To ensure homogeneous dispersion of the DNA:
   a. Allow the DNA to stand at 4°C for several hours after dilution and addition of 10× restriction enzyme buffer.
   b. *Gently* stir the DNA solution from time to time using a sealed glass capillary.
   c. After addition of the restriction enzyme, *gently* stir the solution for 2–3 minutes at 4°C before warming the reaction to the appropriate temperature.
   d. After digestion for 15–30 minutes, add a second aliquot of restriction enzyme and stir the reaction as described above.

   It is important to include controls to show whether digestion with the restriction enzyme(s) is complete and whether transfer and hybridization of the DNA has worked efficiently. This can be accomplished by setting up a series of digests containing mammalian DNA and a very small amount of a plasmid carrying a sequence complementary to the probe (e.g., 10 μg of mammalian DNA and 10^{-5}, 10^{-6}, and 10^{-7} μg of plasmid). During digestion, the plasmid will be cleaved into a series of bands that will be invisible when the gel is examined by staining with ethidium bromide. However, fragments of the correct size should be detected by subsequent hybridization to the probe. These controls should be loaded into wells that lie toward one side of the gel, well away from the test samples of mammalian DNA. This reduces the chances of accidental contamination and minimizes the possibility that the hybridization from the controls will obscure the signal from the test samples.

2. At the end of the digestion, add the appropriate amount of gel-loading buffer (see Appendix B) and separate the fragments of DNA by electrophoresis through an agarose gel (for genomic DNA, a 0.7% gel cast in 0.5× TBE containing ethidium bromide [0.5 μg/ml] may be used; see Chapter 6, pages 6.9–6.13).

Caution: Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when working with solutions that contain this dye. After use, these solutions should be decontaminated by one of the methods described in Appendix E.

It is important to include controls containing DNAs of known size that can serve as molecular-weight standards (e.g., bacteriophage λ DNA cleaved with *Hin*dIII). These marker DNAs are usually run in the two outside wells of the gel. Sufficient marker DNA to be detectable by staining with ethidium bromide (~ 200 ng) should be applied to the gel.

Occasionally, problems arise during loading of the gel because the DNA solution will not sink to the bottom of the well. This occurs when very-high-molecular-weight DNA is present at the end of the digest, for example, when the digest is incomplete or when mammalian DNA has been digested with enzymes such as *Not*I that generate very large fragments of DNA. To minimize the problem, make sure that the DNA is homogeneously dispersed and load the samples very slowly into the wells of the gel. After loading, allow the gel to stand for a few minutes so that the DNA can diffuse evenly throughout the wells.

If the DNA has been stored at 4°C, it should be heated to 56°C for 2–3 minutes before it is applied to the gel. This disrupts any base pairing that may have occurred between protruding cohesive termini.

The gel should be run slowly (<1 V/cm) to prevent smearing of the fragments of DNA. Ethidium bromide (0.5 μg/ml) is usually included in both the gel and the electrophoresis buffer. However, some workers believe that the presence of the dye increases the tendency of bands of high-molecular-weight DNA to "smile" during electrophoresis. They therefore carry out electrophoresis in the absence of ethidium bromide and stain the gel later.

3. After electrophoresis is completed, photograph the gel as described in Chapter 6, page 6.19. Place a transparent ruler alongside the gel so that the distance that any band of DNA has migrated can be read directly from the photographic image.

   If desired, the gel may be stored at this stage before the DNA is denatured and transferred to the filter. Wrap the gel in Saran Wrap and store it on a flat surface at 4°C. Because the bands of DNA diffuse during storage, the gel should not be put aside for more than 1 day before being processed.

TRANSFER OF DNA FROM AGAROSE GELS TO SOLID SUPPORTS

There are three methods to transfer fragments of DNA from agarose gels to solid supports (nitrocellulose filters or nylon membranes):

1. *Capillary transfer.* In the capillary transfer method (Southern 1975), DNA fragments are carried from the gel in a flow of liquid and deposited on the surface of the solid support. The liquid is drawn through the gel by capillary action that is established and maintained by a stack of dry, absorbent paper towels (Figure 9.3). The rate of transfer of the DNA depends on the size of the DNA fragments and the concentration of agarose in the gel. Small fragments of DNA (<1 kb) are transferred almost quantitatively from a 0.7% agarose gel within 1 hour; larger fragments are transferred more slowly and less efficiently. For example, capillary transfer of DNAs greater than 15 kb in length requires at least 18 hours, and even then the transfer is not complete. The efficiency of transfer of large DNA fragments is determined by the fraction of molecules that escape from the gel before it becomes dehydrated. As elution proceeds, fluid is drawn not only from the reservoir, but also from the interstices of the gel itself. This reduces the gel to a rubbery substance through which DNA molecules cannot pass. This problem of dehydration can be partially alleviated by partial hydrolysis of the DNA prior to capillary transfer (Wahl et al. 1979; Meinkoth and Wahl 1984). The DNA in the gel is exposed to weak acid (which results in partial depurination), followed by strong base (which hydrolyzes the phosphodiester backbone at the sites of depurination). The resulting fragments of DNA (~ 1 kb in length) can then be transferred rapidly from the gel with high efficiency. However, it is important not to let the depurination reaction proceed too far; otherwise, the DNA is cleaved into small fragments that are too short to bind efficiently to the solid support. Depurination/hydrolysis can also cause the bands of the final autoradiograph to assume a "fuzzy" appear-

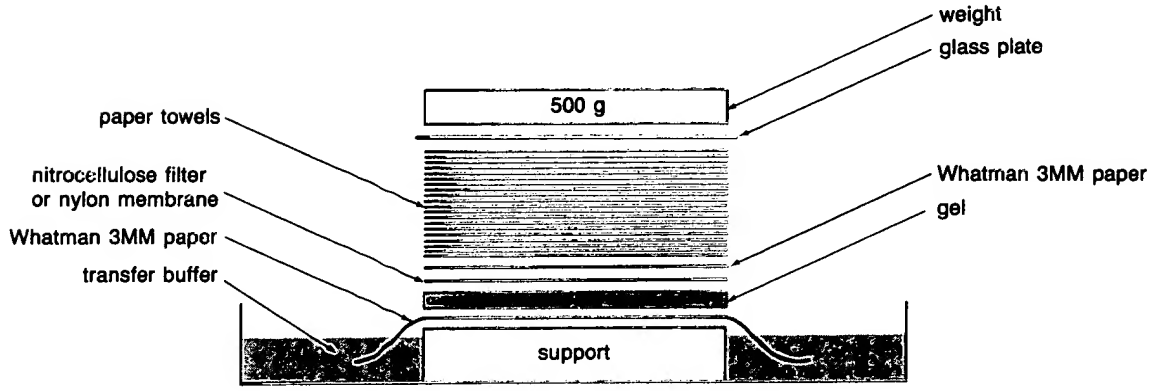

FIGURE 9.3
Capillary transfer of DNA from agarose gels. Buffer is drawn from a reservoir and passes through the gel into a stack of paper towels. The DNA is eluted from the gel by the moving stream of buffer and is deposited on a nitrocellulose filter or nylon membrane. A weight applied to the top of the paper towels helps to ensure a tight connection between the layers of material used in the transfer system.

ance, presumably because of increased diffusion of DNA during transfer. Depurination/hydrolysis is therefore recommended only when it is known ahead of time that the target DNA fragments exceed 15 kb in length.

When the target DNA fragments are present in high concentration (e.g., in restriction digests of plasmids or bacteriophage λ recombinants), the capillary method can be used to transfer DNA simultaneously and rapidly from a single gel to two nitrocellulose filters or nylon membranes (Smith and Summers 1980) (Figure 9.4). The only source of transfer buffer is the liquid trapped in the gel itself, and the efficiency of transfer is therefore relatively poor. This method is not recommended when high sensitivity is required (e.g., detection of single-copy sequences in mammalian DNA), but it is perfectly adequate for Southern analysis of recombinant plasmids and bacteriophage λ DNAs.

2. *Electrophoretic transfer.* This method is not practical when nitrocellulose is used as the solid support because of the high ionic strengths of the buffers that are required to bind nucleic acids to these filters. These buffers conduct electric current very efficiently, and it is necessary to use large volumes to ensure that the buffering power of the system does not become depleted by electrolysis. In addition, extensive external cooling is required to overcome the effects of ohmic heating (Arnheim and Southern 1977). Until recently, therefore, electrophoretic transfer was carried out with supports such as diazobenzyloxymethyl (DBM)- or *o*-aminophenylthioether (APT)-cellulose, which bind nucleic acids efficiently at low ionic strength (see, e.g., Bittner et al. 1980; Stellwag and Dahlberg 1980). Although these types of solid supports are no longer in common use, electrophoretic transfer has undergone a recent resurgence with the advent of charged nylon membranes. Nucleic acids as small as 50 bp will bind to these membranes in buffers of very low ionic strength (Reed and Mann 1985).

Although single-stranded DNA and RNA can be transferred directly, fragments of double-stranded DNA must first be denatured in situ as described on page 9.38. The gel is then neutralized and soaked in

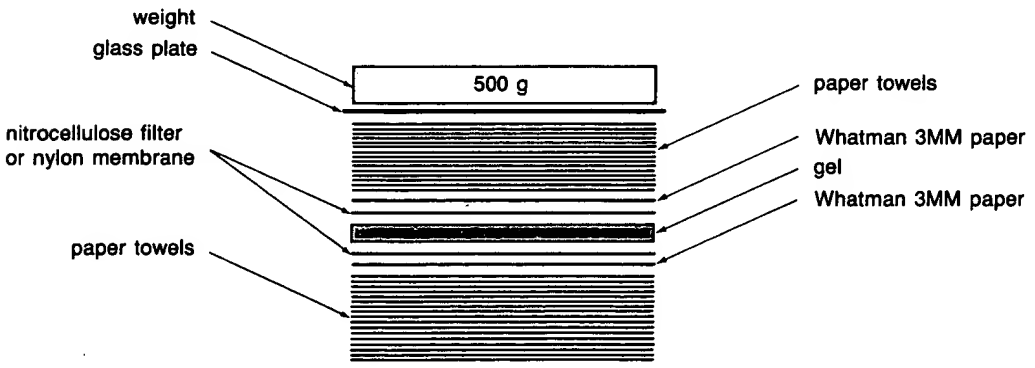

FIGURE 9.4
Capillary transfer of DNA from agarose gels to two solid supports simultaneously.

electrophoresis buffer (1 × TBE; see Appendix B) before being mounted between porous pads aligned between parallel electrodes in a large tank of buffer. The time required for complete transfer depends on the size of the fragments of DNA, the porosity of the gel, and the strength of the applied field. However, because even high-molecular-weight nucleic acids migrate relatively rapidly from the gel, depurination/hydrolysis is unnecessary and transfer is generally complete within 2–3 hours. Because electrophoretic transfer requires comparatively large electric currents, it is often difficult to maintain the electrophoresis buffer at a temperature compatible with efficient transfer of DNA. Many commercially available electrophoretic transfer machines are equipped with cooling devices, but others are effective only when used in a coldroom. Because of these and other problems, we recommend that electrophoretic transfer be used only when transfer by capillary action or under vacuum is inefficient, for example, when it is necessary to analyze fragments of DNA separated by electrophoresis through polyacrylamide gels (Stellwag and Dahlberg 1980; Church and Gilbert 1984).

3. *Vacuum transfer.* DNA and RNA can be transferred rapidly and quantitatively from gels under vacuum. Several vacuum transfer devices are now available commercially in which the gel is placed in contact with a nitrocellulose filter or nylon membrane supported on a porous screen over a vacuum chamber. Buffer, drawn from an upper reservoir, elutes nucleic acids from the gel and deposits them on the filter or membrane.

   Vacuum transfer is more efficient than capillary transfer and is extremely rapid; DNAs that have been partially depurinated and denatured with alkali are quantitatively transferred within 30 minutes from gels of normal thickness (4–5 mm) and normal agarose concentration (<1%). If carried out carefully, vacuum transfer can result in a two- to threefold enhancement of the hybridization signal obtained from Southern transfers (Medveczky et al. 1987; Olszewska and Jones 1988).

   All of the commercially available apparatuses work well as long as care is taken to ensure that the vacuum is applied evenly over the entire surface of the gel. Special care should be taken with the wells of horizontal agarose gels, which tend to break during preparation of the gel for transfer. If this occurs, the wells should be trimmed from the gel before transfer. (The wells need not be trimmed from the gel as long as they are unbroken.) It is also important not to apply too much vacuum during transfer. When the vacuum exceeds 60 cm of water, the gels become compressed and the efficiency of transfer is reduced.

The following types of transfer methods are described below:

- Capillary transfer of DNA to nitrocellulose filters
- Simultaneous transfer of DNA from a single agarose gel to two nitrocellulose filters
- Capillary transfer of DNA to nylon membranes under both neutral and alkaline conditions

We have not given detailed descriptions of the methods used for electrophoretic transfer and vacuum transfer. We recommend that these techniques be carried out according to the instructions provided by the manufacturer of the apparatus that is used.

CAPILLARY TRANSFER OF DNA TO NITROCELLULOSE FILTERS

1. After electrophoresis, transfer the gel to a glass baking dish and trim away any unused areas of the gel with a razor blade. Cut off the bottom left-hand corner of the gel; this serves to orient the gel during the succeeding operations.

2. Denature the DNA by soaking the gel for 45 minutes in several volumes of 1.5 M NaCl, 0.5 N NaOH with constant, *gentle* agitation (e.g., on a rotary platform).

   If the gel floats to the surface of the liquid, weigh it down with several pasteur pipettes.

   If the fragments of interest are larger than approximately 15 kb, transfer may be improved by nicking the DNA by brief depurination prior to denaturation with base. After step 1, soak the gel for 10 minutes in several volumes of 0.2 N HCl and then rinse briefly with deionized water.

3. Rinse the gel briefly in deionized water, and then neutralize it by soaking for 30 minutes in several volumes of a solution of 1 M Tris (pH 7.4), 1.5 M NaCl at room temperature with constant, gentle agitation. Change the neutralization solution and continue soaking the gel for a further 15 minutes.

4. While the gel is in the neutralization solution, wrap a piece of Whatman 3MM paper around a piece of Plexiglas or a stack of glass plates to form a support that is longer and wider than the gel. Place the wrapped support inside a large baking dish. Fill the dish with transfer buffer (10 × SSC or 10 × SSPE) until the level of the liquid reaches almost to the top of the support. When the 3MM paper on the top of the support is thoroughly wet, smooth out all air bubbles with a glass rod.

   20 × SSC or 20 × SSPE can also be used as the transfer buffer. The binding of DNA to nitrocellulose depends on the ionic strength of the transfer buffer (Gillespie and Spiegelman 1965). The smaller the fragments of DNA, the higher the ionic strength required for their efficient retention on the nitrocellulose filter. For Southern transfer of fragments of DNA less than 500 nucleotides in length, 20 × SSC or 20 × SSPE should be used (Nagamine et al. 1980). Alternatively, nylon membranes, which bind small DNA fragments more efficiently than nitrocellulose filters, may be used (see pages 9.42–9.46).

   The efficiency with which small fragments of DNA are retained by nitrocellulose also depends on the pore size of the filter. Fragments less than 300 nucleotides in length are not retained by standard nitrocellulose filters, whose pore size is 0.45 micron. The efficiency of transfer can be improved if nitrocellulose filters with a pore size of 0.2 micron are used.

5. Using a fresh scalpel or a paper cutter, cut a piece of nitrocellulose filter (Schleicher and Schuell BA85 or equivalent) about 1 mm larger than the gel in both dimensions. Use gloves and blunt-ended forceps (e.g., Millipore forceps) to handle the filter. A nitrocellulose filter that has been touched by greasy hands will not wet!

6. Float the nitrocellulose filter on the surface of a dish of deionized water until it wets completely from beneath, and then immerse the filter in transfer buffer for at least 5 minutes. Using a clean scalpel blade, cut a corner from the nitrocellulose filter to match the corner cut from the gel.

   The rate at which different batches of nitrocellulose filters wet varies enormously. If the filter is not saturated after floating for several minutes on water, it should be replaced with a new filter, since the transfer of DNA to an unevenly wetted filter is unreliable. The original filter should not be discarded but should be autoclaved for 5 minutes between pieces of 3MM paper saturated with 2 × SSC. This usually results in complete wetting of the filter. The autoclaved filter, sandwiched between the autoclaved 3MM papers saturated with 2 × SSC, may be stored at 4°C in a sealed plastic bag until it is needed.

7. Remove the gel from the neutralization solution and invert it so that its underside is now uppermost. Place the inverted gel on the support so that it is centered on the wet 3MM papers. Make sure that there are no air bubbles between the 3MM paper and the gel.

8. Surround, but do not cover, the gel with Saran Wrap or Parafilm. This serves as a barrier to prevent liquid from flowing directly from the reservoir to paper towels placed on top of the gel. If these towels are not precisely stacked, they tend to droop over the edge of the gel and may touch the support. This type of short-circuiting is a major cause of inefficient transfer of DNA from the gel to the filter.

9. Place the wet nitrocellulose filter on top of the gel so that the cut corners are aligned. One edge of the filter should extend just over the edge of the line of slots at the top of the gel. Do not move the filter once it has been applied to the surface of the gel. Make sure that there are no air bubbles between the filter and the gel.

10. Wet two pieces of 3MM paper (cut to exactly the same size as the gel) in 2 × SSC and place them on top of the wet nitrocellulose filter. Smooth out any air bubbles with a glass rod.

11. Cut a stack of paper towels (5–8 cm high) just smaller than the 3MM papers. Place the towels on the 3MM papers. Put a glass plate on top of the stack and weigh it down with a 500-g weight (see Figure 9.3). The objective is to set up a flow of liquid from the reservoir through the gel and the nitrocellulose filter, so that fragments of denatured DNA are eluted from the gel and are deposited on the nitrocellulose filter.

12. Allow the transfer of DNA to proceed for 8–24 hours. As the paper towels become wet, they should be replaced.

13. Remove the paper towels and the 3MM papers above the gel. Turn over the gel and the nitrocellulose filter and lay them, gel side up, on a dry sheet of 3MM paper. Mark the positions of the gel slots on the filter with a very-soft-lead pencil or a ballpoint pen.

14. Peel the gel from the filter and discard it. Soak the filter in 6 × SSC for 5 minutes at room temperature. This removes any pieces of agarose sticking to the filter.

To assess the efficiency of transfer of DNA, the gel may be stained for 45 minutes in a solution of ethidium bromide (0.5 μg/ml in water) and examined by ultraviolet illumination. Note that the intensity of fluorescence will be quite low because the DNA remaining in the gel has been denatured.

Cautions: Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when working with solutions that contain this dye. After use, these solutions should be decontaminated by one of the methods described in Appendix E.

Ultraviolet radiation is dangerous, particularly to the eyes. To minimize exposure, make sure that the ultraviolet light source is adequately shielded and wear protective goggles or a full safety mask that efficiently blocks ultraviolet light.

15. Remove the filter from the 6 × SSC and allow excess fluid to drain away. Place the filter flat on a paper towel to dry for at least 30 minutes at room temperature.

16. Sandwich the filter between two sheets of dry 3MM paper. Fix the DNA to the filter by baking for 30 minutes to 2 hours at 80°C in a vacuum oven.

Overbaking can cause the filter to become brittle. If the gel was not completely neutralized before the DNA was transferred, the filter will turn yellow or brown during baking and chip very easily. The background of nonspecific hybridization also increases dramatically.

17. Hybridize the DNA immobilized on the filter to a ^{32}P-labeled probe as described on pages 9.52–9.55.

Any filters not used immediately in hybridization reactions should be wrapped loosely in aluminum foil and stored under vacuum at room temperature.

SIMULTANEOUS TRANSFER OF DNA FROM A SINGLE AGAROSE GEL TO TWO NITROCELLULOSE FILTERS

DNA can be transferred simultaneously from a single agarose gel to two nitrocellulose filters as described by Smith and Summers (1980) (see Figure 9.4).

1. After electrophoresis, prepare the gel for transfer as described in steps 1–3 on page 9.38.

2. After neutralization, soak the gel for 30 minutes in 10 × SSC. Cut the bottom left-hand corner from the gel; this provides a convenient way to orient the gel and the nitrocellulose filters.

3. While the gel is soaking, cut and wet two pieces of nitrocellulose (0.45-micron pore size), first in water and then in 10 × SSC. Cut one corner from each filter.

   To retain small fragments of DNA (< 300 nucleotides), use nitrocellulose filters with a small pore size (0.2 micron).

4. Lay one of the nitrocellulose filters on a piece of dampened 3MM paper. Lay the gel on top of the filter, aligning the cut corner of the gel with the cut corner of the filter. Without delay, cover the gel with the second nitrocellulose filter, followed by another piece of dampened 3MM paper. Make sure that there are no air bubbles between the filters and the gel.

5. Transfer the entire sandwich of 3MM papers, nitrocellulose filters, and gel onto a stack of paper towels. Cover the sandwich with a second stack of paper towels. Put a glass plate on top of the entire stack and weigh it down with a 500-g weight.

6. After 2 hours, remove the nitrocellulose filters, mark the approximate positions of the gel slots with a very-soft-lead pencil or a ballpoint pen, and process them for hybridization as described on page 9.40 (step 14 onward).

Transfer of DNA from Agarose Gels to Nylon Membranes

For almost 20 years, immobilization and hybridization of denatured DNA was carried out exclusively with nitrocellulose—first with powder (Hall and Spiegelman 1961) and then with sheets (Nygaard and Hall 1963; Gillespie and Spiegelman 1965). Despite its evident success, nitrocellulose is not an ideal matrix for solid-phase hybridization. First, nucleic acids are attached by hydrophobic rather than covalent interactions and are released slowly from the matrix during hybridization and washing at high temperatures. Second, nitrocellulose filters become brittle when dry and cannot survive more than one or two cycles of hybridization and washing. In recent years, therefore, a number of alternative supporting matrices have been explored. These include DBM-cellulose (Goldberg et al. 1979; Wahl et al. 1979) and APT-cellulose (Seed 1982a,b), both of which bind DNA and RNA covalently. Nucleic acids attached to these supports do not leach during hybridization or washing at high temperature and can therefore be hybridized to several probes sequentially. However, activated derivatives of cellulose are laborious to synthesize and do not stand up to the rigors of hybridization much better than nitrocellulose itself. This problem has been solved by the introduction of various types of nylon membranes that bind nucleic acids irreversibly and are far more durable than nitrocellulose filters. Immobilized nucleic acids can therefore be hybridized sequentially to several different probes without damaging the membrane. Furthermore, because nucleic acids can be immobilized on nylon in buffers of low ionic strength, transfer of nucleic acids from gels to nylon membranes can be carried out electrophoretically. This can be useful when capillary or vacuum transfer of DNA is inefficient, for example, when fragments of DNA are transferred from polyacrylamide gels. In addition, large membranes can be handled without fear that they will split, tear, or curl. The sole disadvantage of nylon membranes is a tendency to give increased levels of background hybridization, especially with RNA probes. In many cases, this problem can be overcome by using increased amounts of blocking agents in the prehybridization and hybridization steps.

Two basic types of nylon membranes are available commercially—unmodified nylon and charge-modified nylon. Although both types of membranes can bind nucleic acids, the latter is preferred for transfer and hybridization, since its positively charged surface has a greater capacity for binding nucleic acids. Many different types of charged nylon membranes are available that vary in the type of charge, the method used to apply it, and the density of the nylon mesh. Both double- and single-stranded nucleic acids may be applied to nylon membranes in a wide range of solvents (retention is quantitative in solvents as diverse as water, 0.25 N HCl, and 0.4 N NaOH). However, each manufacturer provides specific instructions for the transfer of nucleic acids to their particular type of charged nylon membrane. These instructions should be followed exactly, since they presumably have been shown to yield the best results. (For practical information about the use of nylon membranes, see Reed and Mann [1985].) The general protocol given below works well with most types of positively charged nylon membranes but may not be optimal for any particular brand.

Nylon membranes must be treated to immobilize the DNA after it has been transferred. The DNA becomes fixed to the nylon membrane if it is thorough-

ly dried (e.g., under vacuum) or if it is exposed to low doses of ultraviolet irradiation (254 nm) after transfer of the nucleic acid. The latter method, although a nuisance to set up, is preferred since it is much quicker and the nucleic acid becomes covalently attached to the membrane. In addition, there are reports that DNA transferred to nylon membranes in buffers of high ionic strength and immobilized with ultraviolet irradiation yields greatly enhanced hybridization signals (Khandjian 1987).

Hybridization of radiolabeled probes to nucleic acids immobilized on nylon membranes is carried out essentially as described on pages 9.52–9.55 for nitrocellulose filters. Either formamide or aqueous solvents may be used, and background binding of the probe may be suppressed by including either 1% SDS, Denhardt's reagent, or 0.05 × BLOTTO.

CAPILLARY TRANSFER OF DNA TO NYLON MEMBRANES UNDER NEUTRAL CONDITIONS

1. Fractionate the DNA by gel electrophoresis, and process the gel as described in steps 1–3 on page 9.38.

2. While the gel is in the neutralization solution, prepare the nylon membrane as follows:

   a. Using a fresh scalpel or a paper cutter, cut a piece of membrane about 1 mm larger than the gel in both dimensions. Use gloves and blunt-ended forceps (e.g., Millipore forceps) to handle the membrane.

   b. Float the membrane on the surface of a dish of deionized water until it wets completely from beneath, and then immerse the membrane in transfer buffer (10 × SSC or 10 × SSPE) for at least 5 minutes. Using a clean scalpel blade, cut a corner from the membrane to match the corner cut from the gel.

3. Transfer the denatured DNA from the gel to the membrane by capillary action as described in steps 7–15 on pages 9.39–9.40.

4. To fix the DNA to the membrane either

   - place the dried membrane between two pieces of 3MM paper, and bake the membrane for 30 minutes to 2 hours at 80°C in a vacuum or conventional oven, or
   - expose the side of the membrane carrying the DNA to a source of ultraviolet irradiation (254 nm).

   Caution: Ultraviolet radiation is dangerous, particularly to the eyes. To minimize exposure, make sure that the ultraviolet light source is adequately shielded and wear protective goggles or a full safety mask that efficiently blocks ultraviolet light.

   Immobilization of nucleic acids by ultraviolet irradiation can greatly enhance the hybridization signal obtained with some brands of positively charged nylon membranes (Khandjian 1987). However, for maximum effect, it is important to make sure that the membrane is not overirradiated. The aim is to form cross-links between a small fraction of the thymine residues in the DNA and positively charged amine groups on the surface of the membrane (Church and Gilbert 1984). Overirradiation results in the covalent attachment of a high proportion of the thymines, with consequent decrease in hybridization signal. The side of the membrane carrying the DNA should face the ultraviolet light source. Most manufacturers advise that damp membranes should be exposed to a total of 1.5 J/sq. cm and that dry membranes should be exposed to 0.15 J/sq. cm. However, we recommend carrying out a series of preliminary experiments to determine empirically the amount of irradiation required to produce the maximum hybridization signal.

If the membrane is not to be used immediately in hybridization experiments, it should be wrapped loosely in aluminum foil and stored under vacuum at room temperature.

CAPILLARY TRANSFER OF DNA TO NYLON MEMBRANES UNDER ALKALINE CONDITIONS

The ability of positively charged nylon membranes to bind denatured DNA under alkaline conditions has advantages in some circumstances, for example, when using probes that are complementary to inverted repeat sequences. However, the level of background hybridization is almost always significantly higher when the membrane has been exposed to high concentrations of alkali for extended periods of time. This problem can often be overcome by increasing the concentration of blocking agents in the hybridization step.

1. After electrophoresis, transfer the gel to a glass baking dish and trim away any unused areas of the gel with a razor blade. Cut off the bottom left-hand corner of the gel; this serves to orient the gel during the succeeding operations.

2. Denature the DNA by soaking the gel for 15 minutes in denaturation/transfer solution (0.4 N NaOH, 1 M NaCl) with constant, *gentle* agitation (e.g., on a rotary platform). Change the solution and continue to soak the gel for a further 20 minutes with gentle agitation.

   If the gel floats to the surface of the liquid, weigh it down with several pasteur pipettes.

   If the fragments of interest are larger than approximately 15 kb, transfer may be improved by nicking the DNA by brief depurination prior to denaturation with base. After step 1, soak the gel for 10 minutes in several volumes of 0.2 N HCl and then rinse briefly with deionized water.

3. While the gel is soaking in the denaturation/transfer solution, prepare the nylon membrane as follows:

   a. Using a fresh scalpel or a paper cutter, cut a piece of membrane about 1 mm larger than the gel in both dimensions. Use gloves and blunt-ended forceps (e.g., Millipore forceps) to handle the membrane.

   b. Float the membrane on the surface of a dish of deionized water until it wets completely from beneath, and then immerse the membrane in denaturation/transfer solution for at least 5 minutes. Using a clean scalpel blade, cut a corner from the membrane to match the corner cut from the gel.

4. Transfer the denatured DNA from the gel to the membrane by capillary action as described in steps 7–13 on page 9.39.

5. Peel the gel from the membrane and discard it. Soak the membrane in 0.5 M Tris · Cl (pH 7.2), 1 M NaCl for 15 minutes at room temperature. This neutralizes the membrane and removes any pieces of agarose sticking to it.

   To assess the efficiency of transfer of DNA, the gel may be stained for 45 minutes in a solution of ethidium bromide (0.5 μg/ml in 0.1 M Tris · Cl [pH 7.2]) and examined by ultraviolet illumination. Note that the intensity of fluorescence will be quite low because the DNA remaining in the gel has been denatured.

Caution: Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when working with solutions that contain this dye. After use, these solutions should be decontaminated by one of the methods described in Appendix E.

6. Remove the membrane from the neutralizing solution and allow excess fluid to drain away. Place the membrane flat on a paper towel to dry for at least 30 minutes at room temperature.

7. To fix the DNA to the membrane either

   - place the dried membrane between two pieces of 3MM paper, and bake the membrane for 30 minutes to 2 hours at 80°C in a vacuum or conventional oven, or
   - expose the side of the membrane carrying the DNA to a source of ultraviolet irradiation (254 nm).

     Caution: Ultraviolet radiation is dangerous, particularly to the eyes. To minimize exposure, make sure that the ultraviolet light source is adequately shielded and wear protective goggles or a full safety mask that efficiently blocks ultraviolet light.

     Immobilization of nucleic acids by ultraviolet irradiation can greatly enhance the hybridization signal obtained with some brands of positively charged nylon membranes (Khandjian 1987). However, for maximum effect, it is important to make sure that the membrane is not overirradiated. The aim is to form cross-links between a small fraction of the thymine residues in the DNA and positively charged amine groups on the surface of the membrane (Church and Gilbert 1984). Overirradiation results in the covalent attachment of a high proportion of the thymines, with consequent decrease in hybridization signal. The side of the membrane carrying the DNA should face the ultraviolet light source. Most manufacturers advise that damp membranes should be exposed to a total of 1.5 J/sq. cm and that dry membranes should be exposed to 0.15 J/sq. cm. However, we recommend carrying out a series of preliminary experiments to determine empirically the amount of irradiation required to produce the maximum hybridization signal.

If the membrane is not to be used immediately in hybridization experiments, it should be wrapped loosely in aluminum foil and stored under vacuum at room temperature.

When hybridizing membranes that have been exposed to alkali, use the higher concentrations of blocking agents described on page 9.48, point 5.

HYBRIDIZATION OF RADIOLABELED PROBES TO IMMOBILIZED NUCLEIC ACIDS

There are many methods available to hybridize radioactive probes in solution to nucleic acids immobilized on solid supports such as nitrocellulose filters or nylon membranes. These methods differ in the following respects:

- Solvent and temperature used (e.g., 68°C in aqueous solution or 42°C in 50% formamide)
- Volume of solvent and length of hybridization (large volumes for periods as long as 3 days or minimal volumes for periods as short as 4 hours)
- Degree and method of agitation (continuous shaking or stationary)
- Use of agents such as Denhardt's reagent or BLOTTO to block the non-specific attachment of the probe to the surface of the solid matrix
- Concentration of the labeled probe and its specific activity
- Use of compounds, such as dextran sulfate (Wahl et al. 1979) or polyethylene glycol (Renz and Kurz 1984; Amasino 1986), that increase the rate of reassociation of nucleic acids
- Stringency of washing following the hybridization

Although the choice depends to a large extent on personal preference, we offer the following guidelines for choosing among the various methods available.

1. Hybridization reactions in 50% formamide at 42°C are less harsh on nitrocellulose filters than is hybridization at 68°C in aqueous solution. However, it has been found that the kinetics of hybridization in 80% formamide are approximately four times slower than in aqueous solution (Casey and Davidson 1977). Assuming a linear relationship between the rate of hybridization and the formamide concentration, the rate in 50% formamide should be two to three times slower than the rate in aqueous solution. Both types of solvents give excellent results and neither has a clear-cut advantage over the other.
2. The smaller the volume of hybridization solution, the better. In small volumes of solution, the kinetics of nucleic acid reassociation are faster and the amount of probe needed can be reduced so that the DNA on the filter acts as the driver for the reaction. However, it is essential that sufficient liquid be present for the filters to remain covered at all times by a film of the hybridization solution.
3. Continual movement of the probe solution across the filter is unnecessary, even for a reaction driven by the DNA immobilized on the filter. However, if a large number of filters are hybridized simultaneously, agitation is advisable to prevent the filters from adhering to one another.
4. The kinetics of the hybridization reaction are difficult to predict from theoretical considerations, partly because the exact concentration of the

immobilized nucleic acid and its availability for hybridization are unknown. When using probes that have the capacity to self-anneal (e.g., nick-translated double-stranded DNA), the following rule of thumb is useful: Allow the hybridization to proceed for a time sufficient to enable the probe in solution to achieve $1-3 \times C_o t_{1/2}$. In 10 ml of hybridization solution, 1 μg of a probe of 5-kb complexity will reach $C_o t_{1/2}$ in 2 hours. To determine the time of half-renaturation for any other probe, simply enter the appropriate values into the following equation:

$$1/x \times y/5 \times z/10 \times 2 = \text{number of hours to achieve } C_o t_{1/2}$$

where x = the weight of the probe added (in micrograms), y = its complexity (for most probes, complexity is proportional to the length of the probe in kilobases), and z = the volume of the reaction (in milliliters).

After hybridization to $3 \times C_o t_{1/2}$ has been reached, the amount of probe available for additional hybridization to the filter is negligible. For probes that do not have the capacity to self-anneal (e.g., RNA probes synthesized in vitro by bacteriophage-encoded DNA-dependent RNA polymerases; see Chapter 10), the hybridization time may be shortened, since the lack of a competing reaction in the solution favors hybridization of the probe to the DNA immobilized on the filter.

5. Several different types of agents can be used to block the nonspecific attachment of the probe to the surface of the filter. These include Denhardt's reagent (Denhardt 1966), heparin (Singh and Jones 1984), and nonfat dried milk (Johnson et al. 1984). Frequently, these agents are used in combination with denatured, fragmented salmon sperm or yeast DNA and detergents such as SDS. In our experience, virtually complete suppression of background hybridization is obtained by prehybridizing filters with a blocking agent consisting of 5 × Denhardt's reagent, 0.5% SDS, and 100 μg/ml denatured, fragmented DNA. We recommend this mixture whenever the signal-to-noise ratio is expected to be low, for example, when carrying out northern analysis of low-abundance mRNAs or Southern hybridizations with single-copy sequences of mammalian DNA. However, in most other circumstances (Grunstein/Hogness hybridization [1975], Benton/Davis hybridization [1977], Southern hybridization [1975] of abundant DNA sequences, etc.), we recommend using 0.25% nonfat dried milk (0.05 × BLOTTO; Johnson et al. 1984). This is much less expensive, easier to use than Denhardt's reagent, and, for these purposes, gives results that are equally satisfactory. In general, Denhardt's reagent is more effective for nylon membranes. The signal-to-noise ratio obtained with most brands of nylon membranes is higher with Denhardt's reagent than with BLOTTO. Nonfat dried milk is not recommended when using RNA probes or when carrying out northern hybridizations because of the possibility that it might contain unacceptably high levels of RNAase activity. For more information about blocking agents, see Table 9.1.

6. Blocking agents are usually included in both the prehybridization and hybridization solutions when nitrocellulose filters are used. However, when the nucleic acid is immobilized on nylon membranes, the blocking agents are often omitted from the hybridization solution, since high

TABLE 9.1 Blocking Agents Used to Suppress Background in Hybridization Experiments

Agent	Recommended uses
Denhardt's reagent	northern hybridizations hybridizations using RNA probes single-copy Southern hybridizations hybridizations involving DNA immobilized on nylon membranes

Denhardt's reagent (Denhardt 1966) is usually made up as a 50× stock solution, which is filtered and stored at −20°C. The stock solution is diluted tenfold into prehybridization buffer (usually 6× SSC or 6× SSPE containing 0.5% SDS and 100 μg/ml denatured, fragmented salmon sperm DNA). 50× Denhardt's reagent contains 5 g of Ficoll (Type 400, Pharmacia), 5 g of polyvinylpyrrolidone, 5 g of bovine serum albumin (Fraction V; Sigma), and H_2O to 500 ml.

BLOTTO	Grunstein/Hogness hybridization Benton/Davis hybridization all Southern hybridizations other than single-copy dot blots

1× BLOTTO (Bovine Lacto Transfer Technique Optimizer; Johnson et al. 1984) is 5% nonfat dried milk dissolved in water containing 0.02% sodium azide. It should be stored at 4°C and diluted 25-fold into prehybridization buffer before use. BLOTTO should not be used in combination with high concentrations of SDS, which will cause the milk proteins to precipitate. If background hybridization is a problem, NP-40 may be added to the hybridization solution to a final concentration of 1%. BLOTTO should not be used as a blocking agent in northern hybridizations because of the possibility that it might contain unacceptably high levels of RNAase.

Caution: Sodium azide is poisonous. It should be handled with great care, wearing gloves, and solutions containing it should be clearly marked.

Heparin	Southern hybridization in situ hybridization

Heparin (Sigma H-7005 porcine grade II or equivalent) is dissolved at a concentration of 50 mg/ml in 4× SSPE or 4× SSC and stored at 4°C. It is used as a blocking agent at a concentration of 500 μg/ml in hybridization solutions containing dextran sulfate; in hybridization solutions that do not contain dextran sulfate, heparin is used at a concentration of 50 μg/ml (Singh and Jones, 1984).

Denatured, fragmented salmon sperm DNA	Southern and northern hybridizations

Salmon sperm DNA (Sigma type III sodium salt) is dissolved in water at a concentration of 10 mg/ml. If necessary, the solution is stirred on a magnetic stirrer for 2–4 hours at room temperature to help the DNA to dissolve. The concentration of NaCl is adjusted to 0.1 M, and the solution is extracted once with phenol and once with phenol:chloroform. The aqueous phase is recovered, and the DNA is sheared by passing it 12 times rapidly through a 17-gauge hypodermic needle. The DNA is precipitated by adding 2 volumes of ice-cold ethanol. It is then recovered by centrifugation and redissolved at a concentration of 10 mg/ml in water. The OD_{260} of the solution is determined and the exact concentration of the DNA is calculated. The solution is then boiled for 10 minutes and stored at −20°C in small aliquots. Just before use, the solution is heated for 5 minutes in a boiling-water bath and then chilled quickly in ice water. Denatured, fragmented salmon sperm DNA should be used at a concentration of 100 μg/ml in prehybridization solutions.

concentrations of protein are believed to interfere with the annealing of the probe to its target. This quenching of the hybridization signal is particularly noticeable when oligonucleotides or probes less than 100 nucleotides in length are used.

7. In the presence of 10% dextran sulfate or 10% polyethylene glycol, the rate of hybridization is accelerated approximately tenfold (Wahl et al. 1979; Renz and Kurz 1984; Amasino 1986) because nucleic acids are excluded from the volume of the solution occupied by the polymer and their effective concentration is therefore increased. Although dextran sulfate and polyethylene glycol are useful in circumstances where the rate of hybridization is the limiting factor in detecting rare sequences (e.g., northern or genomic Southern blots), they are of no benefit when screening bacterial colonies or bacterial plaques. In addition, they can sometimes lead to high backgrounds, and hybridization solutions containing them are always difficult to handle because of their viscosity. We therefore recommend that dextran sulfate and polyethylene glycol not be used unless the rate of hybridization is very slow, the filter contains very small amounts of DNA, or the amount of radiolabeled probe is limiting.

8. To maximize the rate of annealing of the probe with its target, hybridizations are usually carried out in solutions of high ionic strength (6 × SSC or 6 × SSPE) at a temperature that is 20–25°C below the melting temperature (T_m). Both solutions work equally well when hybridization is carried out in aqueous solvents. However, when formamide is included in the hybridization buffer, 6 × SSPE is preferred because of its greater buffering power.

9. In general, the washing conditions should be as stringent as possible (i.e., a combination of temperature and salt concentration should be chosen that is approximately 12–20°C below the calculated T_m of the hybrid under study). The temperature and salt conditions can often be determined empirically in preliminary experiments in which samples of genomic DNA immobilized on filters are hybridized to the probe of interest and then washed under conditions of different stringencies.

10. To minimize background problems, it is best to hybridize for the shortest possible time using the minimum amount of probe. For Southern hybridization of mammalian genomic DNA where each lane of the gel contains 10 μg of DNA, 10–20 ng/ml radiolabeled probe (sp. act. = 10^9 cpm/μg or greater) should be used and hybridization should be carried out for 12–16 hours at 68°C in aqueous solution or for 24 hours at 42°C in 50% formamide. For Southern hybridization of fragments of cloned DNA where each band of the restriction digest contains 10 ng of DNA or more, much less probe is required. Typically, hybridization is carried out for 6–8 hours using 1–2 ng/ml radiolabeled probe (sp. act. = 10^9 cpm/μg or greater).

11. *Useful facts*:

    a. The T_m of the hybrid formed between the probe and its target may be estimated from the following equation (Bolton and McCarthy 1962):

$$T_m = 81.5°C + 16.6(\log_{10}[Na^+]) + 0.41(\text{fraction G + C}) - 0.63(\%\ \text{formamide}) - (600/l)$$

where l = the length of the hybrid in base pairs.

This equation is valid for:

- Concentrations of Na^+ in the range of 0.01 M to 0.4 M. It predicts T_m less accurately in solutions of higher $[Na^+]$.
- DNAs whose G + C content is in the range of 30% to 75%. Note that the depression of T_m in solutions containing formamide is greater for poly(dA:dT) (0.75°C/1% formamide) and less for DNAs rich in poly(dG:dC) (0.50°C/1% formamide) (Casey and Davidson 1977).

The equation applies to the "reversible" T_m that is defined by optical measurement of hyperchromicity at OD_{257}. The "irreversible" T_m, which is more important for autoradiographic detection of DNA hybrids, is usually 7–10°C higher than that predicted by the equation.

Similar equations have been derived for:

i. RNA probes hybridizing to immobilized RNA (Bodkin and Knudson 1985)

$$T_m = 79.8°C + 18.5(\log_{10}[Na^+]) + 0.58(\text{fraction G + C}) + 11.8(\text{fraction G + C})^2 - 0.35(\%\ \text{formamide}) - (820/l)$$

ii. DNA:RNA hybrids (Casey and Davidson 1977)

$$T_m = 79.8°C + 18.5(\log_{10}[Na^+]) + 0.58(\text{fraction G + C}) + 11.8(\text{fraction G + C})^2 - 0.50(\%\ \text{formamide}) - (820/l)$$

Comparison of these equations shows that the relative stability of nucleic acid hybrids decreases in the following order: RNA:RNA (most stable), RNA:DNA (less stable), and DNA:DNA (least stable). In aqueous solutions, the T_m of a DNA:DNA hybrid is approximately 10°C lower than that of the equivalent RNA:RNA hybrid. In 80% formamide, the T_m of an RNA:DNA hybrid is approximately 10°C higher than that of the equivalent DNA:DNA hybrid.

b. The T_m of a double-stranded DNA decreases by 1–1.5°C with every 1% decrease in homology (Bonner et al. 1973).

The above equations apply only to hybrids greater than 100 nucleotides in length. The behavior of oligonucleotide probes is described in detail in Chapter 11.

For a general discussion of hybridization of nucleic acids bound to solid supports, see Meinkoth and Wahl (1984).

Hybridization of Radiolabeled Probes to Nucleic Acids Immobilized on Nitrocellulose Filters or Nylon Membranes

Although the method given below deals with RNA or DNA immobilized on nitrocellulose filters, only slight modifications are required to adapt the procedure to nylon membranes. These modifications are noted at the appropriate places in the text.

1. Prepare the prehybridization solution appropriate for the task at hand. Approximately 0.2 ml of prehybridization solution will be required for each square centimeter of nitrocellulose filter or nylon membrane.

   The prehybridization solution should be filtered through a 0.45-micron disposable cellulose acetate filter (Schleicher and Schuell Uniflow syringe filter No. 57240 or equivalent).

Prehybridization solutions

For detection of low-abundance sequences:

Either
6 × SSC (or 6 × SSPE)
5 × Denhardt's reagent
0.5% SDS
100 μg/ml denatured, fragmented salmon sperm DNA

or
6 × SSC (or 6 × SSPE)
5 × Denhardt's reagent
0.5% SDS
100 μg/ml denatured, fragmented salmon sperm DNA
50% formamide

For preparation of Denhardt's reagent and denatured, fragmented salmon sperm DNA, see Table 9.1.

Formamide: Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by stirring on a magnetic stirrer with Dowex XG8 mixed-bed resin for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C.

For detection of moderate- or high-abundance sequences:

Either
6 × SSC (or 6 × SSPE)
0.05 × BLOTTO

or
6 × SSC (or 6 × SSPE)
0.05 × BLOTTO
50% formamide

For preparation of BLOTTO, see Table 9.1.

When ^{32}P-labeled cDNA or RNA is used as a probe, poly(A)$^+$ RNA at a concentration of 1 μg/ml may be included in the prehybridization and hybridization solutions to prevent the probe from binding to T-rich sequences that are found fairly commonly in eukaryotic DNA.

2. Float the nitrocellulose filter or nylon membrane containing the target DNA on the surface of a tray of 6 × SSC (or 6 × SSPE) until it becomes thoroughly wetted from beneath. Submerge the filter for 2 minutes.

3. Slip the wet filter into a heat-sealable bag (e.g., Sears Seal-A-Meal or equivalent). Add 0.2 ml of prehybridization solution for each square centimeter of nitrocellulose filter or nylon membrane.

   Squeeze as much air as possible from the bag. Seal the open end of the bag with the heat sealer. Incubate the bag for 1–2 hours submerged at the appropriate temperature (68°C for aqueous solvents; 42°C for solvents containing 50% formamide).

   Often, small bubbles of air form on the surface of the filter as the temperature of the prehybridization solution increases. It is important that these bubbles be removed by occasionally agitating the fluid in the bag; otherwise, the components of the prehybridization solution will not be able to coat the filter evenly. This problem can be minimized by heating the prehybridization solution to the appropriate temperature before adding it to the bag.

4. If the radiolabeled probe is double-stranded, denature it by heating for 5 minutes at 100°C. Single-stranded probe need not be denatured. Chill the probe rapidly in ice water.

   Alternatively, the probe may be denatured by adding 0.1 volume of 3 N NaOH. After 5 minutes at room temperature, transfer the probe to ice water and add 0.05 volume of 1 M Tris · Cl (pH 7.2) and 0.1 volume of 3 N HCl. Store the probe in ice water until it is needed.

   For Southern hybridization of mammalian genomic DNA where each lane of the gel contains 10 μg of DNA, 10–20 ng/ml radiolabeled probe (sp. act. = 10^9 cpm/μg or greater) should be used. For Southern hybridization of fragments of cloned DNA where each band of the restriction digest contains 10 ng of DNA or more, much less probe is required. Typically, hybridization is carried out for 6–8 hours using 1–2 ng/ml radiolabeled probe (sp. act. = 10^9 cpm/μg or greater).

5. Working quickly, remove the bag containing the filter from the water bath. Open the bag by cutting off one corner with scissors. Add the denatured probe to the prehybridization solution, and then squeeze as much air as possible from the bag. Reseal the bag with the heat sealer so that as few bubbles as possible are trapped in the bag. To avoid radioactive contamination of the water bath, the resealed bag should be sealed inside a second, noncontaminated bag.

   When using nylon membranes, the prehybridization solution should be *completely* removed from the bag and immediately replaced with hybridization solution. The probe is then added and the bag is resealed.

Hybridization solution for nylon membranes

6 × SSC (or 6 × SSPE)
0.5% SDS
100 μg/ml denatured, fragmented salmon sperm DNA
50% formamide (if hybridization is to be carried out at 42°C)

6. Incubate the bag submerged in a water bath set at the appropriate temperature for the required period of hybridization.

7. Wearing gloves, remove the bag from the water bath and immediately cut off one corner. Pour out the hybridization solution into a container suitable for disposal, and then cut the bag along the length of three sides. Remove the filter and immediately submerge it in a tray containing several hundred milliliters of 2 × SSC and 0.5% SDS at room temperature.

   Important: Do not allow the filter to dry out at any stage during the washing procedure.

8. After 5 minutes, transfer the filter to a fresh tray containing several hundred milliliters of 2 × SSC and 0.1% SDS and incubate for 15 minutes at room temperature with occasional gentle agitation.

   If short oligonucleotides are used as probes, washing should be carried out only for brief periods (1–2 minutes) at the appropriate temperature. For a discussion of the stability of hybrids involving oligonucleotides, see Chapter 11.

9. Transfer the filter to a flat-bottom plastic box containing several hundred milliliters of fresh 0.1 × SSC and 0.5% SDS. Incubate the filter for 30 minutes to 1 hour at 37°C with gentle agitation.

10. Replace the solution with fresh 0.1 × SSC and 0.5% SDS, and transfer the box to a water bath set at 68°C for an equal period of time. Monitor the amount of radioactivity on the filter using a hand-held minimonitor. The parts of the filter that do not contain DNA should not emit a detectable signal. You should not expect to pick up a signal on the minimonitor from filters containing mammalian DNA that has been hybridized to single-copy probes.

11. Briefly wash the filter with 0.1 × SSC at room temperature. Remove most of the liquid from the filter by placing it on a pad of paper towels.

12. Place the damp filter on a sheet of Saran Wrap. Apply adhesive dot labels marked with radioactive ink to several asymmetric locations on the Saran Wrap. These markers serve to align the autoradiograph with the filter. Cover the labels with Scotch Tape. This prevents contamination of the film holder or intensifying screen with the radioactive ink.

    Radioactive ink is made by mixing a small amount of ^{32}P with waterproof black drawing ink. We find it convenient to make the ink in three grades: very hot

(>2000 cps on a hand-held minimonitor), hot (>500 cps on a hand-held minimonitor), and cool (>50 cps on a hand-held minimonitor). Use a fiber-tip pen to apply ink of the desired hotness to the adhesive labels. Attach radioactive-warning tape to the pen, and store it in an appropriate place.

13. Cover the filter with a second sheet of Saran Wrap, and expose the filter to X-ray film (Kodak XAR-2 or equivalent) to obtain an autoradiographic image (see Appendix E). The exposure time should be determined empirically. However, single-copy sequences in mammalian genomic DNA can usually be detected after 16–24 hours of exposure at −70°C with an intensifying screen.

Hybridization of Radiolabeled Oligonucleotides to Genomic DNA

Oligonucleotide probes as short as 17 nucleotides in length may be used to detect single-copy sequences in restriction digests of eukaryotic genomic DNA that have been transferred to solid supports. As discussed in Chapter 11, hybrids of this length are stable enough to be detected in practice only if they are perfectly matched. Duplexes with a single base-pair mismatch are significantly less stable and dissociate at a lower temperature than their perfectly matched counterparts (Wallace et al. 1979; Ikuta et al. 1987). It has therefore been possible to use oligonucleotides of defined sequence to probe fetal DNA for the presence of specific point mutations that cause conditions such as sickle-cell anemia (Conner et al. 1983), certain thalassemias (Orkin et al. 1983; Pirastu et al. 1983), and α_1-antitrypsin deficiency (Kidd et al. 1983); to screen DNA extracted from tumor cells for mutations in oncogenes (Bos et al. 1984, 1985, 1987; Forrester et al. 1987; Rodenhuis et al. 1987); and to analyze highly polymorphic loci, for example, the major histocompatibility complex class I genes (Geliebter et al. 1986).

The methods used when hybridizing with oligonucleotide probes are similar to those described earlier in this chapter. However, attention should be paid to the following points:

1. Because of the small size of the target sequence, a minimum of 30 μg of mammalian genomic DNA should be applied to each lane of the agarose gel.
2. The sequences of oligonucleotides used as probes should be long enough to be unique within the target genome (17 nucleotides for the mammalian genome) and short enough to allow the detection of mismatches under the conditions of hybridization used. Typically, oligonucleotides used for screening mammalian genomic DNA are 19–21 nucleotides in length.
3. When used to detect point mutations, oligonucleotides are used in pairs; one member of the pair is perfectly homologous to the mutated gene sequence and the other is homologous to the wild-type sequence. Usually, the members of the pair differ in sequence by only one nucleotide. Before embarking on an analysis of genomic DNA with these probes, it is essential to establish hybridization and washing conditions using cloned fragments of DNA of known sequence that are homologous to each member of the pair of oligonucleotides. These methods are discussed in detail in Chapter 11. Reconstruction experiments, in which known amounts of the control DNAs are added to a large excess of genomic DNA (at least 30 μg), are then used to test the sensitivity of the system.
4. Oligonucleotides are radiolabeled by [γ-^{32}P]ATP and bacteriophage T4 polynucleotide kinase (see Chapter 11). These probes tend to hybridize nonspecifically to high-molecular-weight DNA immobilized on nitrocellulose filters or nylon membranes, producing a smear toward the top of the autoradiograph. It is therefore important to choose a restriction enzyme (or a combination of restriction enzymes) that yields a hybridizing fragment whose size is not greater than 5 kb.

5. After electrophoresis, the fragments of genomic DNA may be transferred to a solid support by the conventional Southern transfer technique or immobilized within the agarose gel itself by dehydration (Studencki and Wallace 1984). Although DNA immobilized within the gel appears to give somewhat stronger hybridization signals than DNA attached to a solid support, it cannot be hybridized sequentially to many different probes. This is a severe disadvantage when the amount of genomic DNA is limited (as is often the case in prenatal diagnosis, for example). We therefore recommend that the genomic DNA be transferred to a nylon membrane such as Nytran (Schleicher and Schuell) or GeneScreen (du Pont).

6. Wherever possible, negative and positive hybridization controls should be included in each gel.

7. Oligonucleotides may also be used to detect rare transcripts in northern blots that contain 30 μg of total cellular RNA (Zeff et al. 1986) or 5 μg of poly(A)$^+$ RNA (Geliebter et al. 1986).

Removal of Radiolabeled Probes from Nitrocellulose Filters and Nylon Membranes

Probes become irreversibly bound if nitrocellulose filters and nylon membranes are allowed to dry. Therefore, every effort should be made to ensure that the solid supports remain wet at all stages during hybridization, washing, and exposure to X-ray film.

REMOVING PROBES FROM NITROCELLULOSE FILTERS

1. Heat several hundred milliliters of 0.05 × SSC, 0.01 M EDTA (pH 8.0) to boiling. Remove the fluid from the heat and add SDS to a final concentration of 0.1%. Immerse the filter in the hot elution buffer for 15 minutes.

2. Repeat step **1** with a fresh batch of boiling elution buffer.
   Important: Do not allow the filter to dry when transferring it between batches of hot elution buffer.

3. Rinse the filter briefly in 0.01 × SSC at room temperature. Remove most of the liquid from the filter by placing it on a pad of paper towels.

4. Sandwich the damp filter between two sheets of Saran Wrap, and apply it to X-ray film to check that all of the probe has been removed.

5. The filter may now be dried, wrapped loosely in aluminum foil, and stored under vacuum at room temperature until needed.

REMOVING PROBES FROM NYLON MEMBRANES

1. Either
   - immerse the membrane in several hundred milliliters of 1 mM Tris · Cl (pH 8.0), 1 mM EDTA (pH 8.0), 0.1 × Denhardt's reagent (see Table 9.1) for 2 hours at 75°C, or
   - immerse the membrane in 50% formamide, 2 × SSPE for 1 hour at 65°C.

2. Rinse the membrane briefly with 0.1 × SSPE at room temperature. Remove most of the liquid from the membrane by placing it on a pad of paper towels.

3. Sandwich the damp membrane between two sheets of Saran Wrap, and apply it to X-ray film to check that all of the probe has been removed.

4. The membrane may now be dried, wrapped loosely in aluminum foil, and stored under vacuum at room temperature until needed.

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